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Neutral Proteases of Human Polymorphonuclear Granulocytes: Putative Mediators of Pulmonary Damage

by John K. Spitznagel,* Malcolm C. Modrzakowski,* Katherine B. Pryzwansky,* and Edith K. MacRae*

Tissue proteolytic enzymes are currently believed to be critical to the pathogenesis of panacinar emphysema. Polymorphonuclear leukocytes (Polys) have several enzymes including elastase and cathepsin G in their azurophil granules. They have collagenase in their specific granules. We have found that this collagenase is doubly latent. It has the lysosomal type of latency that depends on the impermeability of the unit membrane that surrounds each specific granule. In addition it has a latency that is converted to activity by proteolytic enzymes. The cathepsin G of the azurophil granule is a potent activator of this latent collagenase once the collagenase is released from its membrane dependent latency. Thus latency of enzymes, the nature of the latency and accessibility of the latent enzymes to activating mechanisms must all be taken into account in any analysis of their contribution to pathogenesis of local lung disease. Equally important is the fact that polys are not a prominent cellular component of normal lung. Polys must be attracted to the lung by chemotactic peptides. These peptides must be released by the interaction of inflammatory stimuli, such as smoke particles, with complement components or they must be provided by other sources.

The hypothesis that lung damage in panacinar emphysema is mediated by polys and their proteases is attractive and suggestive evidence supporting this is available. However, more evidence that takes into full account the cell biology of the proteases and poly turnover in the lung are needed to extend the hypothesis and to form a rational basis for therapeutic and prophylactic measures.

Neutrophil polymorphonuclear granulocytes (PMN) and their neutral elastases are among today's leading candidates for principal mediators of chronic obstructive pulmonary disease due to panacinar emphysema (*1*). The concept of these cells and their enzymes as mediators of lung damage fits well with the established anatomical and functional importance of elastic fibers in alveolar septa and respiratory bronchioles. Figure 1 shows the respiratory bronchiole leading into the pulmonary alveoli. Figure 2 shows the numerous elastic fibers woven through the alveolar and bronchiolar walls. This concept also fits with reports that in emphysematous lung elastic fibers have been damaged. Elastase itself affords an element of specificity that enhances the chance that it is important in this picture of damage, since purified collagenases and trypsin seem unable to induce experimental emphysema. As things stand, the most successful animal models of human emphysema depend on intratracheal installation of an elastolytic protease such as papain or elastase from polymorphs. Intravenous injections will work but require much higher doses of enzyme. In hamsters, 0.2-0.5 mg/100 g of elastase by intratracheal installation initiates irreversible lung disease. The exact role of the granulocytes, other than their service as ready sources and vehicles for elastase is still uncertain. However, there appears to be two phases for their participation in lung damage: there is recruitment of the cells and there is exocytosis (degranulation) of the elastase. The recruitment is likely to depend on deposit in respiratory bronchioles and alveoli of foreign particulate matter with cytotoxic materials absorbed onto their surfaces (*2*). Exocytosis of elastase and access of the enzyme to elastin fibers may depend on the direct effects of cytotoxic chemicals

*Departments of Bacteriology and Immunology and Anatomy, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514.

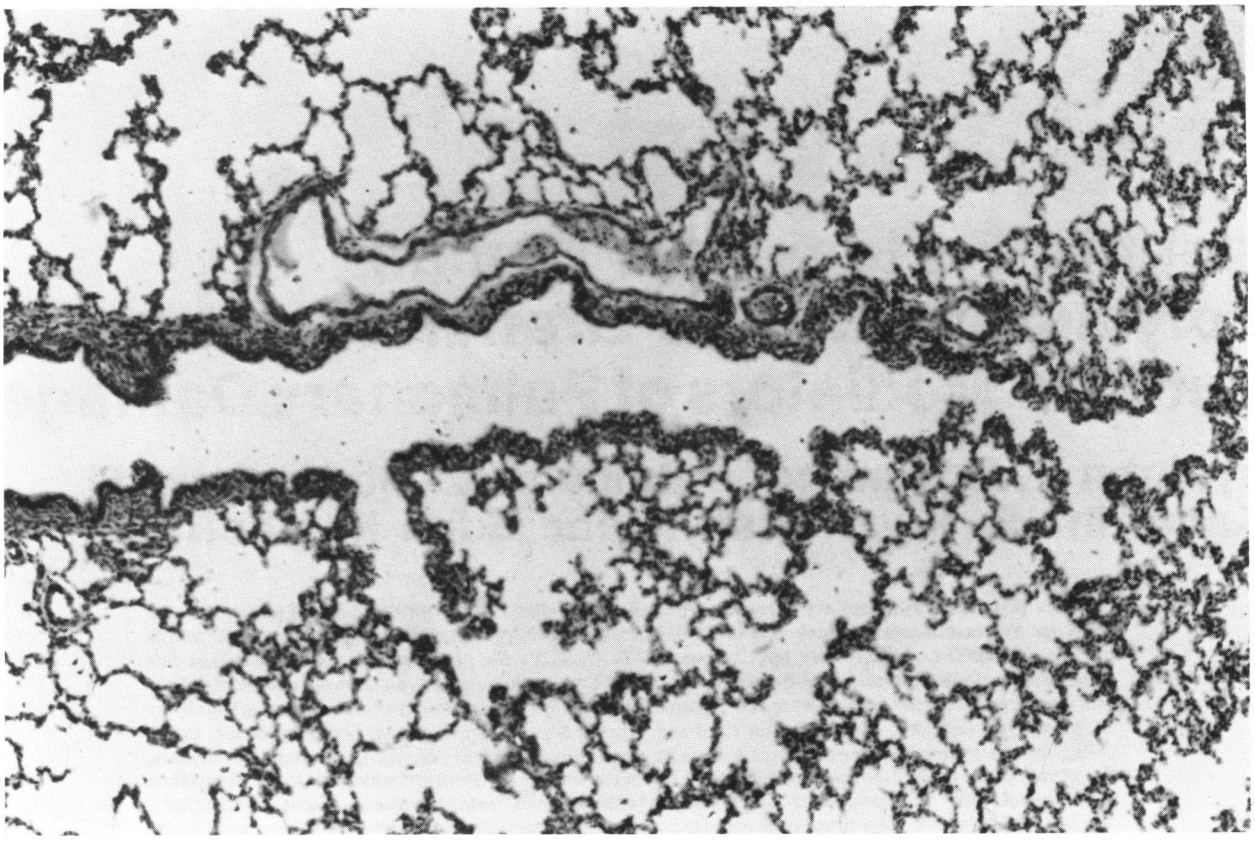

FIGURE 1. Normal bronchiole. Note respiratory bronchiole (left) and alveoli. H and E stained histological section.

that damage the PMN (*3*) and the counterinhibitory effects of these chemicals acting on protease inhibitors from plasma (*4*). Exocytosis of proteases may also be induced with chemicals absorbed to particles phagocytized by the polymorphs.

In view of the rather limited knowledge of the circumstances of local involvement of PMN in the lung, I have chosen not to discuss them but to discuss some pertinent aspects of the cell biology of PMN neutral proteases about which many details are available. I shall try to relate these in a speculative way to the events that may be taking place in the lung. In particular, I shall discuss the kinds of proteases associated with PMN, the way in which they are packaged, the ways in which they come to be released from PMN, and some aspects of their action on connective tissue.

Human PMN have three neutral proteases that have been studied in considerable detail, an elastolytic enzyme, a chymotrypsinlike cathepsin G, and a collagenase (*5-7*). There are, in addition, much less extensively studied enzymes — a plasminogen activator (*8*) and proteinase 3 (P_3) that has hardly been characterized at all (*5*). The elastase and the cathepsin G are serine esterases active against a variety of large molecules such as casein, but the important thing about elastase is its unique capacity to cleave the helical peptide of elastin. Being serine esterases these enzymes are irreversibly inhibited by chloromethyl ketone derivatives of synthetic peptides and by sulfonyl fluorides. The elastase is highly reactive with peptide derivatives that have alanyl-alanyl-prolyl-valyl sequences. Cathepsin G is highly reactive with peptides containing phenylalanine (*7*). The enzymes are readily demonstrated with polyacrylamide gel electrophoresis. They are also inhibited by naturally occurring protease inhibitors found in plasma, α_1-antitrypsin and α_2-macroglobulin, and α_1-antichymotrypsin (*9*). The collagenase is not an esterase; it is unaffected by the chloromethyl ketone inhibitors. Since it is a metalloenzyme, it is inhibited by metal chelators such as ethylendiaminetetraacetic acid (*5*). The synthetic inhibitors are important to studies on the catalytic action of the serine esterases. Moreover, some thought has been given to the possible therapeutic use of the chloromethyl ketone inhibitors to inhibit elastase (*10*). More will be said below about plasma inhibitors.

Returning to the cell biology of the neutral proteases, it is important to know they are carried inside

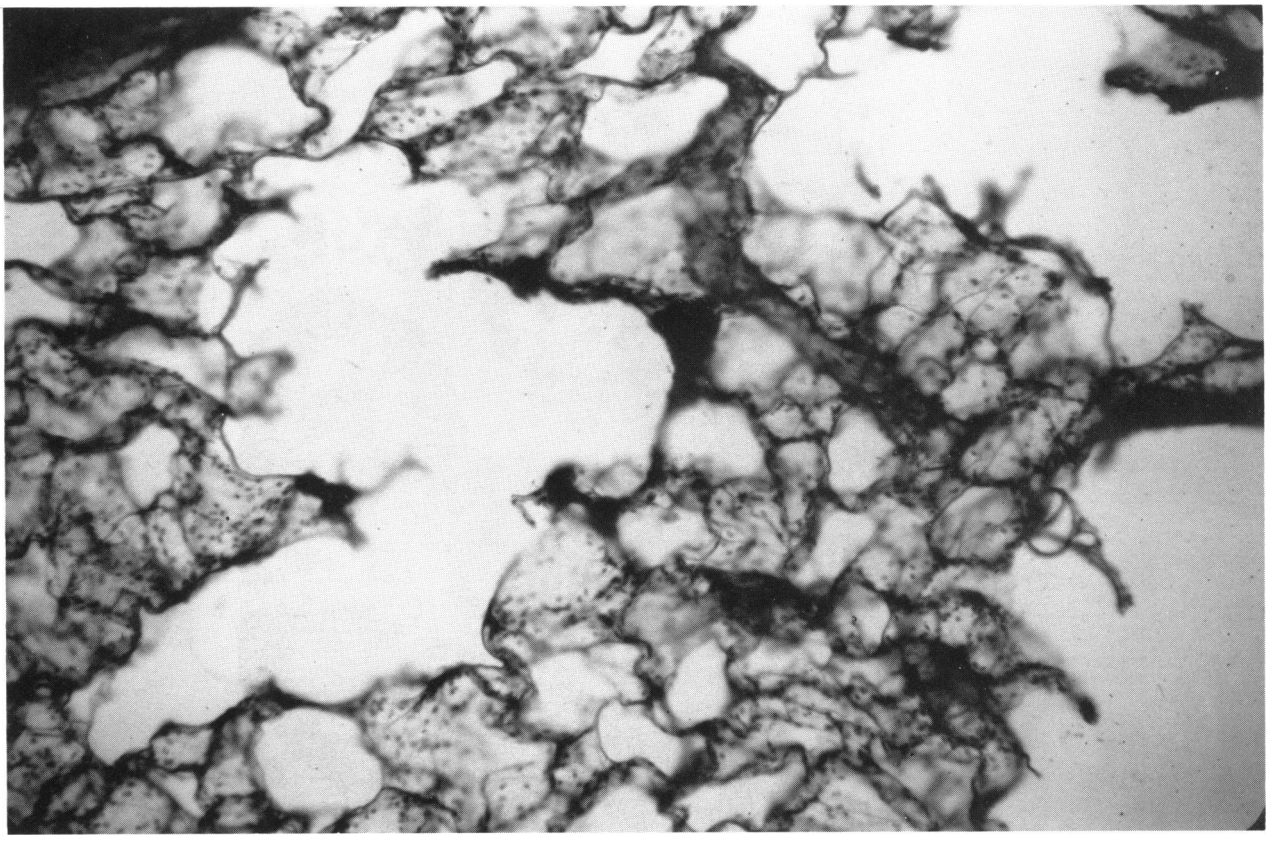

FIGURE 2. Lung. Note elastin fibers about alveoli. Dots are nuclei. Orcein (elastin) stain.

the membranes of granules in the PMN cytoplasm. Figure 3 shows a transmission electron microscope view of the granule apparatus of a resting human PMN. Note that the granules appear heterogeneous; that is, they are of many different sizes and electron densities. In spite of their confusing varieties, there are only two biochemically distinct kinds: the specifics or secondary granules and the azurophils or primary granules. Both granule types are formed while polymorphs are still in the marrow. Azurophils are formed in the promyelocytes and specifics are formed in the myelocytes (*11*). The azurophils tend to be large — about 0.3 μm in diameter. The specifics tend to be smaller — about 0.1 μm in diameter (*12*). The important point is that the elastase and cathepsin G are unequivocally carried in azurophil granules (*5, 6*). Collagenase, however, is in the specifics (*5, 13*). We know this as a result of cell fractionation experiments. Thus purified PMN can be broken up in a homogenizer, their granules released, and the nuclei which contain the antiproteases removed. When this mixture of granules is centrifuged through a sucrose density gradient, several bands containing different subcellular particles form in the gradient. Figure 4 shows a 60 ml centrifuge tube containing such a sucrose density gradient. The subcellular particles have been centrifuged to density equilibrium. The specific granules are seen clearly as band II_f. The azurophil granules are seen as two bands marked III_s and III_f (*12*). When pumped out of the centrifuge tubes and analyzed, these bands of granules are associated uniquely with certain constituents. Table 1 summarizes a few of the constituents of the major granules. The azurophils contain elastase, cathepsin G, and myeloperoxidase (MPO). They also contain acid hydrolases — phosphatase, β-glucuronidase, and lysozyme. The MPO, besides being strongly antibacterial, serves as a unique marker for azurophils (*12*). Most of these enzymes are very cationic proteins. Those in Table 1 are ordered according to their degree of cationicity. The cationic property may promote their binding to connective tissues. The specific granules have lysozyme and in addition they have apo-lactoferrin, an 80,000 Dalton, iron-binding, intracellular analog of transferrin. It forms a specific marker to trace the fate of these granules (*12*). Specific granules also contain a collagenase that is latent as we shall see. Figure 5 shows the results of biochemical and immunochemical analysis of the fractions. The rela-

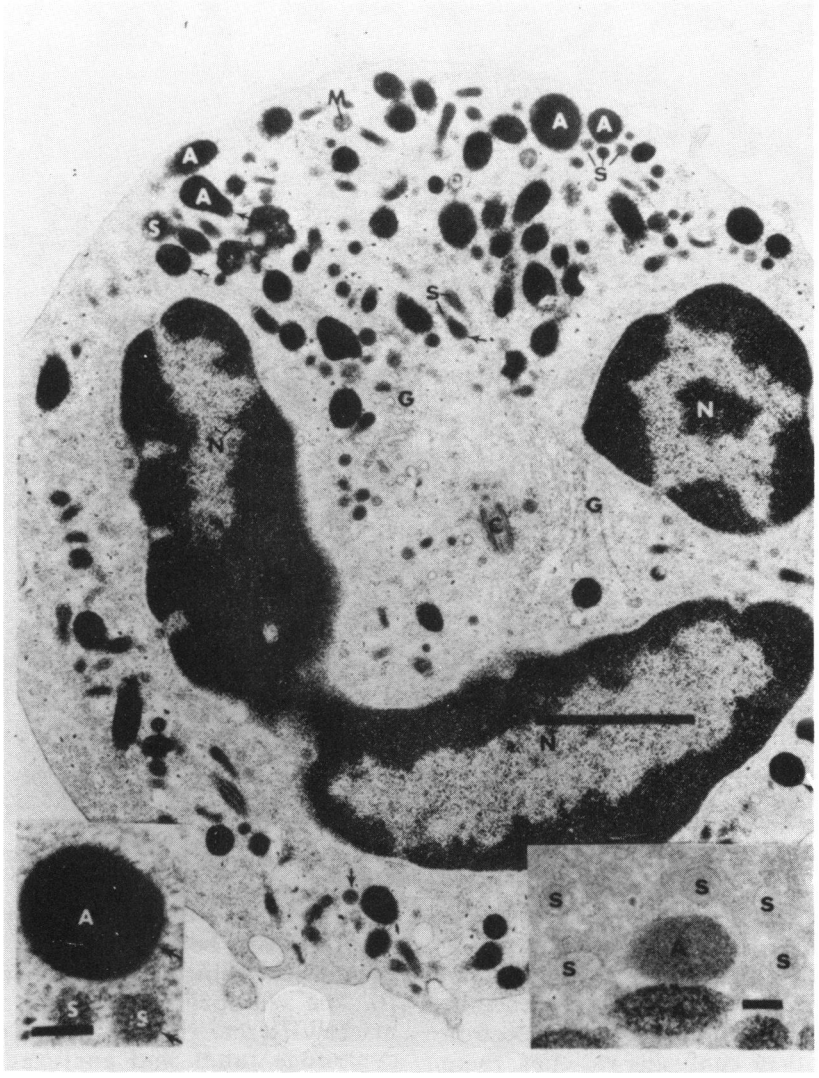

FIGURE 3. Human, normal, resting neutrophil: (A) azurophil granules; (S) specific granules; (M) vestigeal mitochondria; (G) golgi apparatus; (C) centriole; Fixation 0.5% glutaraldehyde, postfixed in osmium tetroxide. Stained with uranyl and lead. Insets: (left) an enlarged detail of figure; (right) granules stained only with peroxidase stain and uranyl. × 30,000.

tive distribution of the enzymes is shown in relation to the gradient in which the particles are depicted as moving from left to right. Enzyme peaks are to be compared with the positions of the various granule populations marked by daggers and by the MPO in the azurophils and the lactoferrin in the specifics. The elastase is distributed exactly as are the azurophil granules. Not shown here but with exactly the same distribution in the azurophil granules is cathepsin G (7). From this we conclude that both elastase and cathepsin G are azurophil granule enzymes.

It can also be seen that the collagenase is in the specific granules. Curiously, the collagenase is doubly latent, that is, it cannot be detected in intact granules. Moreover, it cannot be detected when the specific granule membranes have been removed unless it is activated by a proteolytic enzyme. There is an expected latency in all granule enzymes. It is due to the membranes that surround the granule enzymes rendering them inaccessible to substrate until they are treated to remove the membrane barrier as was done here to measure the MPO, elastase, and cathepsin G. Latency could not be eliminated so simply with the collagenase. The small white histogram shows the small activity detected with only membrane removal. The larger black histogram

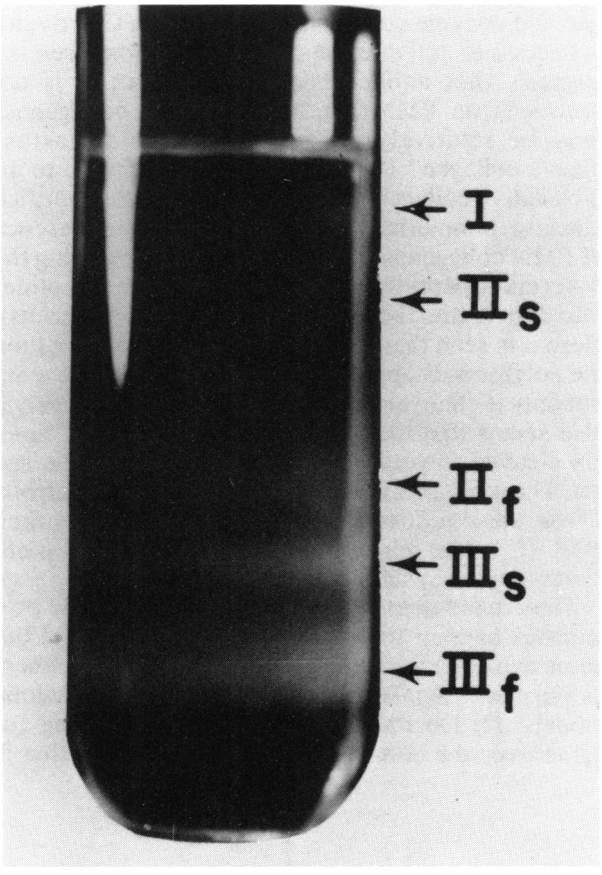

FIGURE 4. Human neutrophil polymorphonuclear granulocyte granules separated on sucrose density gradient. The II_f band comprises the specific granules. III_s and III_f comprise two subclasses of azurophil granules. I, cell membranes; II_s mitochondria.

Table 1. Some constituents of the major granules of neutrophil polymorphonuclear granulocytes.

Azurophil (primary) granules	Specific (secondary) granules
Acid phosphatase	Lysozyme
Acid β-glucuronidase	Lactoferrin
Myeloperoxidase	Collagenolytic enzyme
Elastase	
Lysozyme	
Cathepsin G	

shows the activity and distribution of collagenase with membrane removed and latent enzyme activated by another protease. Trypsin was the first protease we found that could activate the collagenase.

The most effective collagenase activator turned out to be the highly purified cathepsin G of azurophil granules. Elastase of azurophil granules was less effective than cathepsin G or trypsin. This was

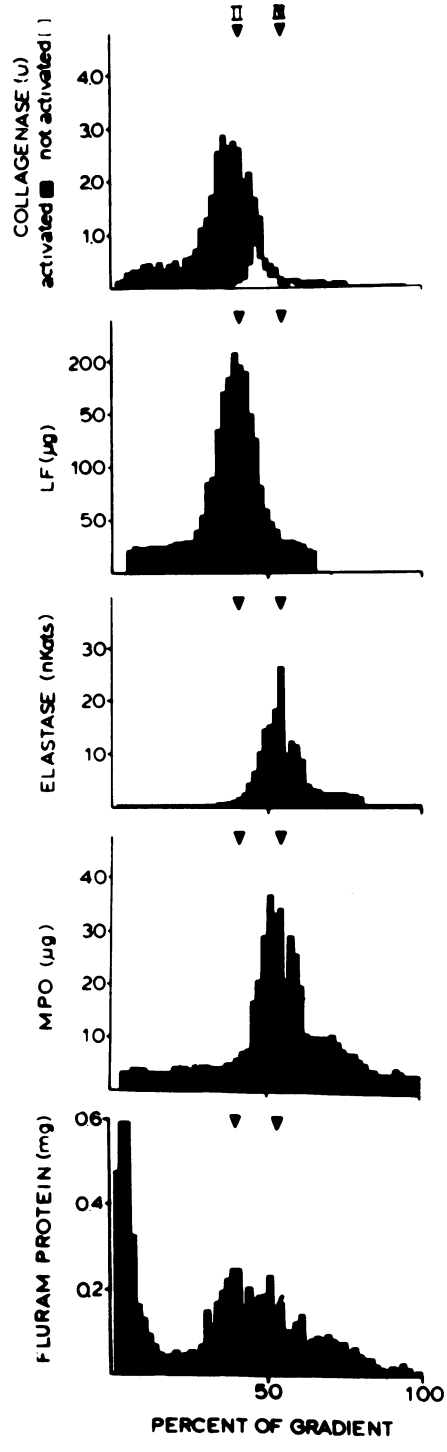

FIGURE 5. Histogram showing activities of collagenase (white histogram latent, black histogram collagenase with trypsin activation), lactoferrin (specific granule marker), myeloperoxidase (azurophil granule marker), elastase, and amounts of protein from sucrose density gradient similar to Figure 4.

shown with the digestion of salt soluble guinea pig skin collagen and was measured with release from the collagen of biologically incorporated ^{14}C-labeled glycine and proline. The most active enzyme was cathepsin G. Trypsin activity was intermediate and elastase was least active.

Now, why is there so much discussion of PMN collagenase if elastase and elastin are the most important factors in lung damage leading to emphysema? Actually, there is also evidence that lung collagen is damaged as well, especially in experimental emphysema (*4*). Hence, a mediator of that damage should be sought. For that reason I shall compare effects of collagenase and elastase on collagen and discuss further the relationships between latency of collagenase and the fate of cathepsin G, its activator. Figure 6 is from sodium dodecyl sulfate-polyacrylamide gel analysis of digestion fragments cleaved from collagen by highly purified samples of these enzymes. Collagenase cleaves the helical peptides and gives rise to the characteristic ¾ and ¼ length fragments seen in gels 3, 4, 5, and 6. The presence of other cleavage products suggests that other enzymes capable of cleaving the peptides are present as well. The amount of fragments increases with time of digestion. The other gels are from collagen and enzyme controls. The cathepsin G activator is needed to activate the collagenase. Some reports suggest, that unlike elastase, cathepsin G is not secreted from PMN (*14*). If this is so, the collagenase may be relatively unimportant. Could elastase cleave collagen? It has been believed unable to attack native collagen, but Figure 7 shows that purified elastase monmerizes native collagen in the presence of PMN collagenase. It could do this by cleaving the *N*-terminal peptides that connect the helical peptides into dimers (the β-chains) and trimers (the γ-chains). Here it is seen that with increasing time of digestion the polymers disappear; no small fragments are seen, but only α-chain monomers remain. Quantitatively it also seems that the α-chains eventually, with time, are cleaved to very small peptide as seen in the last gel. The other gels are collagen and enzyme controls. These observations are in agreement with Starkey et al. (*15*). The relative contributions of the two enzymes in our system remains to be defined.

The crucial question, of course, is how these proteinases happen to emerge from the PMN and become available to attach to elastic and collagen fibers as purified elastase has been made to do in animal models (*1*). Do PMN die and autolyze leaking enzyme over the connective tissue? Evidently that is

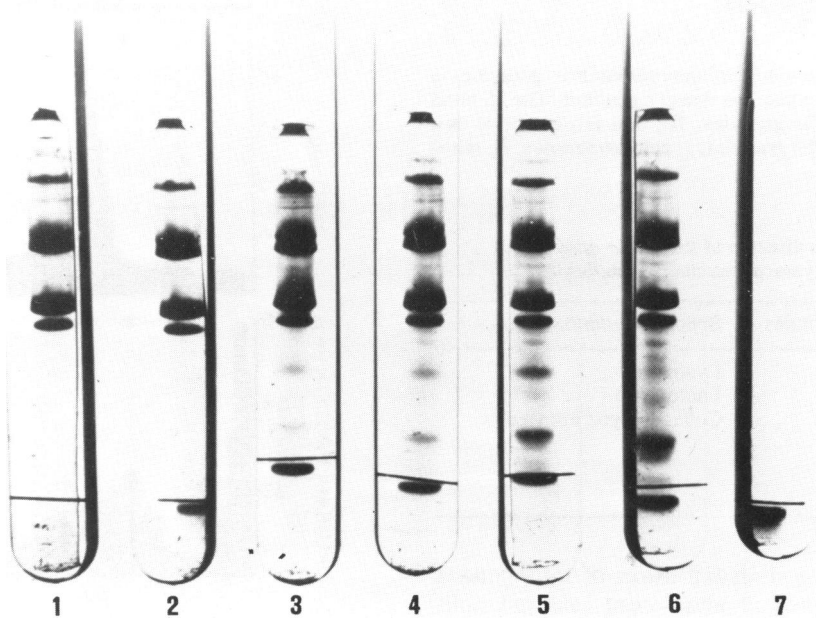

FIGURE 6. Cleavage products of guinea pig skin salt soluble collagen reacted with specific granule collagenase: (1) collagen control; (2) collagen plus granule-activated granule protein, zero time; (3) 0.5 hr; (4) 1 hr; (5) 2 hr; (6) 4 hr with activated enzyme; (7) gel granule enzyme control. The collagenase in this instance was activated by cathepsin G which does not attack native collagen. The cathepsin G was highly purified. The collagenase was used as specific granule extract. Note progressive appearance of α_A (¾) and α_B (¼) fragments. Other cleavage products not identified appeared with progression of time.

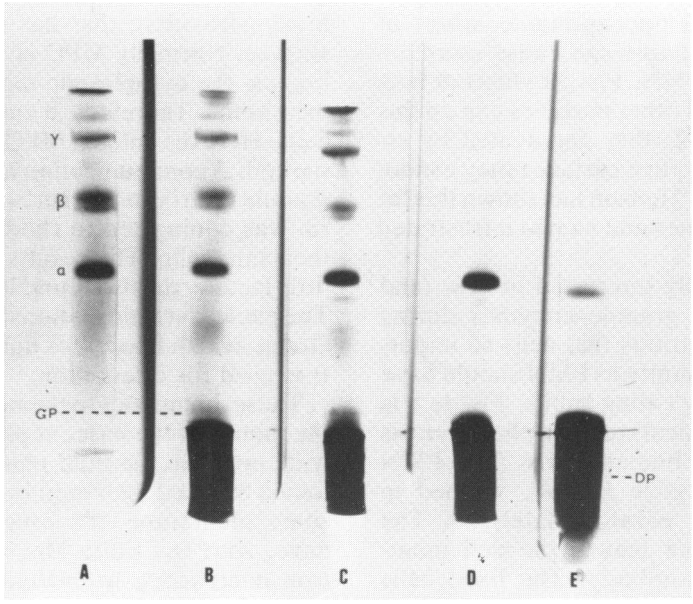

FIGURE 7. Cleavage fragments of collagen digested with azurophil granule elastase for elastase cleavage of salt-soluble guinea pig skin collagen: (A) control collagen; (B) elastase (gp) plus collagen at zero time; (*C*), (*D*), and (*E*) time course of digestion 0.5, 1, and 2 hr incubation. Note disappearance of all but α chains.

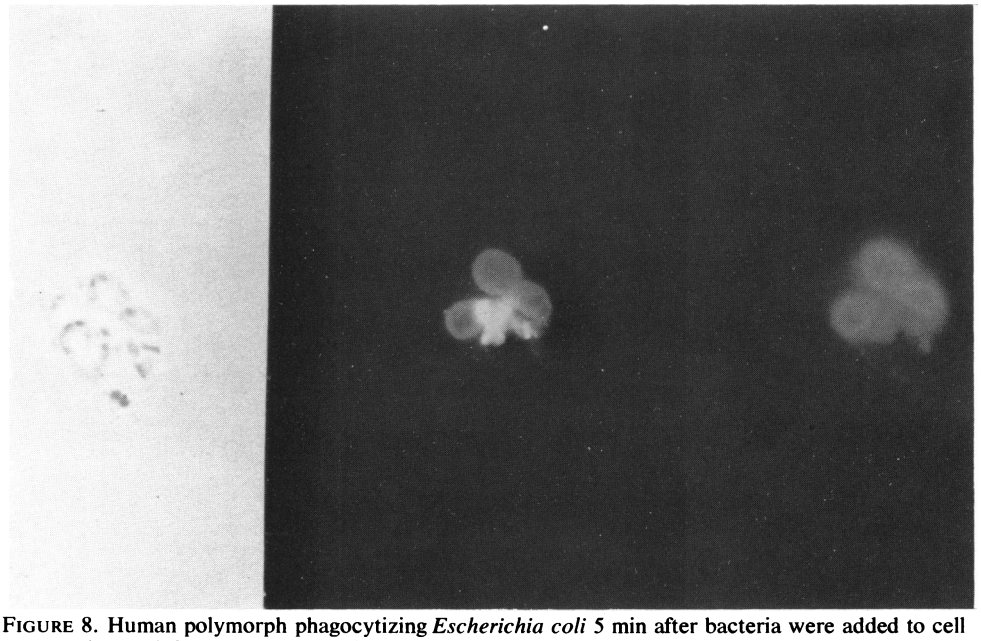

FIGURE 8. Human polymorph phagocytizing *Escherichia coli* 5 min after bacteria were added to cell monolayer: (left) phase contrast view showing bacteria just within a space visable between nuclear lobes; (middle) the fluorescein conjugate of anti-human myeloperoxidase shows that myeloperoxidase has emerged onto the external surface of the cell membrane; (right) double staining with a rhodamine conjugate of anti-human lactoferrin shows that a small amount of lactoferrin is also on the cell surface. The same cell is seen in all three panels. It was fixed with 1% paraformaldehyde. This fixative leaves the cell membrane impermeable to the immunoglobulins of the fluorescent conjugates. Thus only antigen on the outside of the cell surface is stained.

unnecessary. Phagocytosis of particles as small as 1 μm in diameter, given appropriate coatings of immunoglobulins, for example can cause secretion of elastase from human PMN. Phagocytosis of bacteria, yeast cell walls, and other particles can do this as well. This leakage is further aggravated by attempts of PMN to phagocytize surfaces they cannot surround with membrane. Henson has shown this for other PMN granule enzymes and named it frustrated phagocytosis (*16*).

We have been especially interested in how (and also why) PMN secrete granule enzymes during phagocytosis. It seems curious that cells so important in anti-infectious immunity as PMN should have survived with such untidy eating habits. Figure 8 is from an immunocytochemical study on phagocytosis of *Escherichia coli* with human PMN. The PMN were allowed to phagocytize *E. coli,* washed in saline, and fixed in 1% paraformaldehyde. The paraformaldehyde fixative leaves the cell membranes impermeable by antibody. The fixed cells were reacted with antibody against myeloperoxidase conjugated to fluorescein. Any fluorescein bound to the cells indicates antibody specifically bound to myeloperoxidase that has emerged onto the PMN surface. Normally MPO is buried behind two membranes, the cytoplasmic membrane and the granule membrane. Therefore, it cannot be stained in resting cells. Here the antigen MPO is on the outer surface of the cell. A communication has been established from granule matrix to cell surface. Antibody to lactoferrin was conjugated to rhodamine and used to stain the same cells. The results in Figure 8 show that a little lactoferrin also found its way to the cell surface. The nuclear staining is nonspecific and due to Methyl Green, which fluoresces dark red in ultraviolet light. It is used for orientation.

These immunocytochemical results suggest that the mouth of the nascent phagolysosome was open, wide open, to the fluid phase and that the granules joined or fused their membranes with the phagolysosome membrane. To investigate this further we have, with Dr. Edith MacRae, used scanning electron microscopy to evaluate events on the phagocytizing cell surface. Figure 9 shows that indeed the phagolysosome where you see *E. coli* about to enter,

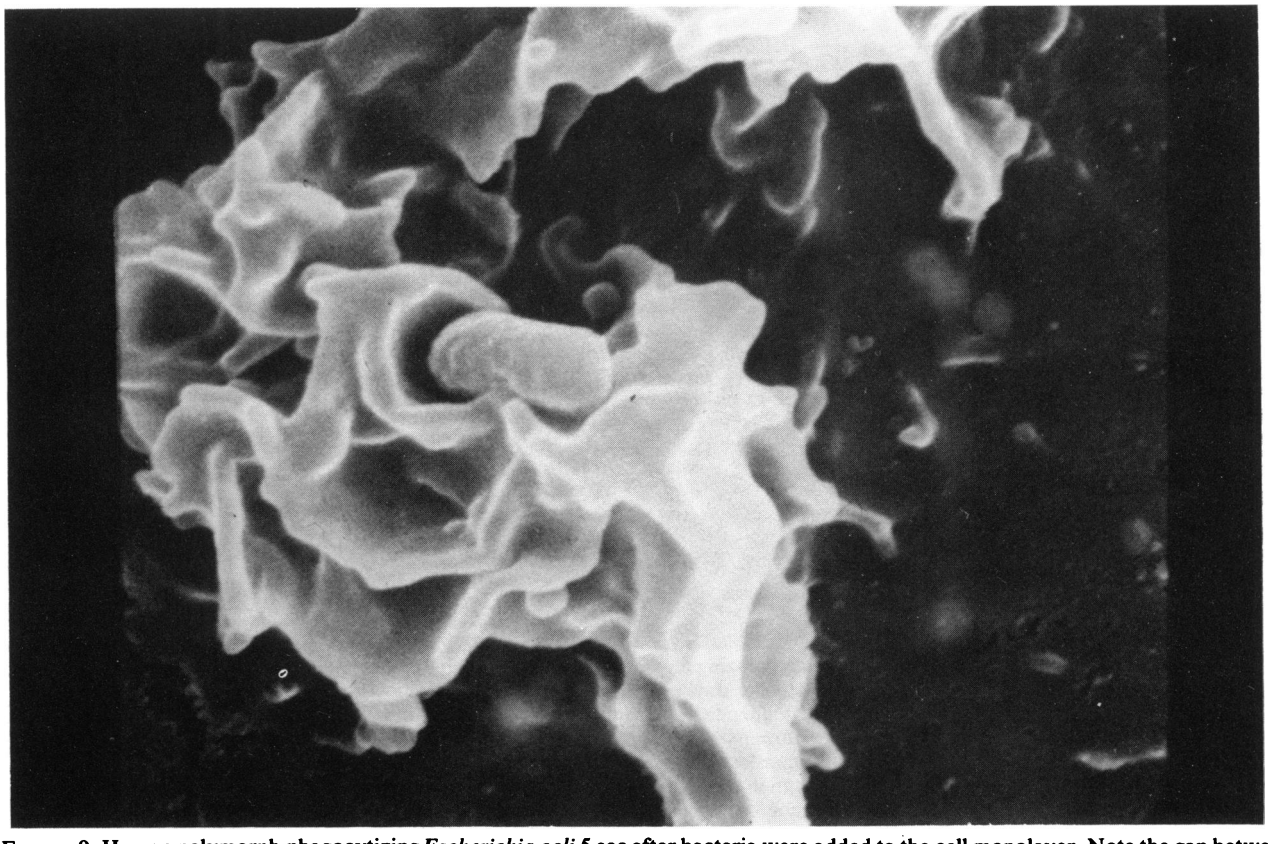

FIGURE 9. Human polymorph phagocytizing *Escherichia coli* 5 sec after bacteria were added to the cell monolayer. Note the gap between the lips of the nascent phagolysosome and the bacterial envelope.

does gape widely offering ample opportunity for exocytosis of the enzymes from the phagolysosome.

Findings from our laboratory and from the literature lead us to conclude that the PMN have potent enzymes capable of digesting both elastic fibers and collagen fibers. These enzymes do leave the cells, but whether they react with the connective tissues and alter them remains to be shown. It is also clear that phagocytosis and stimulation with several soluble mediators and chemicals may also induce release (*3*). Smoke particles with toxic chemicals absorbed to their surfaces are reported to be chemotactic and to stimulate phagocytosis (*2*). Even in the normal mode of antimicrobial phagocytosis granule enzymes are exocytosed onto the PMN surface and they can dissolve in the fluid phase bathing the cells. The azurophil granule contents containing elastase may conceivably within suitable localized spaces evade inhibition by α_1-antitrypsin inhibitor and by α_2-macroglobulin. If this happens, the enzyme could then bind to available elastic fibers within the space and damage them. Whether the collagenase or the procollagenase of specific granules is activated extracellularly under these circumstances is not known. Even if collagenase has no role, the elastase is now known to depolymerize collagen (see above). This depolymerization would be expected to weaken the collagen (*15*).

If proteolytic enzymes generally tend to be exocytosed by phagocytizing PMN why do they not cause damage at all times? For example, why doesn't the PMN response in pneumococcal pneumonia autolyze the lung? It is probably because biological control is available perhaps through the antiproteases of plasma (*9*), perhaps through mechanisms yet to be discovered. Most important among known mechanisms with respect to PMN neutral proteases are plasma α_1-antitrypsin and α_2-macroglobulin. But, there are in addition plasma α_1-antichymotrypsin, plasma β_1-anticollagenase, and plasma antileukoprotease. That α_1-antitrypsin may indeed afford critical control and prevent proteolytic damage to lungs is suggested by the studies on the relationships between homozygous α_1-antitrypsin deficiency and pulmonary emphysema. Finally it should be noted there are antiproteases in bronchial secretions as well as in plasma.

Experimental results recently reported from Janoff's laboratory (*4*) suggests that cigarette smoke concentrate interferes with antiprotease activity due to pure human serum α_1 antitrypsin, challenged with pure human PMN elastase and porcine pancreatic elastase. Moreover, Galdston et al. (*17*) have reported that pack-years of smoking, elastaselike esterolytic activity of polymorphs plus trypsin inhibitory activity of plasma seem to account for 68% of variability in pulmonary function tests observed in MZ and ZZ α_1-antitrypsin phenotypes with chronic obstructive pulmonary disease. One must conclude that the evidence that proteolytic enzymes could be factors in pulmonary damage is highly suggestive, whether these enzymes are from PMN remains to be seen.

Summary

The principal need is for direct, rigorous demonstration that PMN, charged with elastase, are recruited to the strategic part of pulmonary structure in sufficient numbers and with appropriate kinetics to account for low grade but progressive long-term damage to connective tissue — especially to elastin and possibly to collagen. Moreover, it will be necessary to show the nature and existence of conditions that lead to exocytosis of the proteases from polymorphs, that lead to the failure of biological controls due to antiproteases, and that promote degradative enzyme interactions with connective tissue fibers.

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